seminars in **CANCER BIOLOGY**, Vol. 11, 2001: pp. 239–244
doi:10.1006/scbi.2000.0370, available online at http://www.idealibrary.com on IDEAL®

Editorial
The genetics and pathology of mouse mammary cancer

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The first inbred mouse strain having a high incidence of mammary tumors was developed more than six decades ago at the Jackson Laboratory in Bar Harbor. Bittner's demonstration in 1936 of the 'milk factor' in mouse strains having a high incidence of mammary tumors led to the discovery of the mouse mammary tumor virus (MMTV) and cellular proto-oncogenes that are activated by juxtaposed MMTV proviruses. Techniques of experimental mouse genetics developed over the past two decades provided the impetus to specifically study the role of individual proteins in development and cancer. The first transgenic mouse that developed mammary tumors as a result of the expression of a human oncogene in mammary epithelium (the Harvard oncomouse) was established seventeen years ago by Philip Leder and colleagues.[1] Three years later the Leder team demonstrated synergism between oncoproteins, and thus set the stage for the cooperative and multi-event cancer model.[2] These experiments are milestones in breast cancer research, and they ushered in a research era that uses experimental mouse genetics to establish and dissect molecular pathways of breast cancer. While studies in tissue culture cells permit the molecular dissection of pathways operative in a homogeneous population of cells under experimental conditions, research in mice integrates the complexity of an organ and its different cell types in the context of the dynamic hormonal and physiological status of the animal.

Mouse models have been heralded as a breakthrough for the identification of biochemical pathways that control normal cell growth and tumorigenesis, and thus as a tool to develop and test diagnostic and therapeutic regimen. This clearly is a long-term goal. In the foreseeable future, experimental mouse genetics will help to define signaling pathways in the mammary gland, and by that token understand the ground rules that govern the life and death of a mammary cell. However, we need to appreciate that fundamental species differences may complicate the mission to develop mouse models that accurately replicate human breast cancer. Such species differences are exemplified in Brca1. While one mutated Brca1 allele is sufficient to cause breast and ovarian cancer in women, mammary cancer in the mouse is only seen after the inactivation of both alleles.[3]

Recently, an entire Review Issue of *Oncogene* was dedicated to 'Mouse Models for Breast Cancer' (*Oncogene*, vol. 19, no. 8, February 2000). Thus, the focus of this Editorial will be on the challenges we face using current transgenic and gene knock-out technologies, on the pathobiology of available mouse models and on newly developed CD-ROM and web-based tools that can aid the researcher to evaluate their particular mouse model. A CD-ROM entitled, 'Mammary Cancer in Humans and Mice: A Tutorial for Comparative Pathology' accompanies this Editorial (see inside back cover). This CD-ROM was designed to provide the newcomer to mammary biology a source of histopathology images of the common lesions found in the human breast and the mouse mammary gland.

The mice at hand

Four types of conceptually distinct mouse models have been developed and used to address defined questions. First, the forced constitutive expression of a gene, whose protein product could be expected to autonomously promote cell proliferation and transformation. Second the temporally defined and reversible expression of genes to investigate processes of tumor progression. Third, the forced expression of proteins that do not promote proliferation and transformation by themselves, but exhibit

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1044–579X / 01 / 030239+ 06 / $35.00 / 0

auxiliary functions in combination with oncogenes. Fourth, the inactivation of genes that may control tumor suppression, cell survival and growth and differentiation pathways.

Conventional transgenesis

The classic transgenic approach addresses the fundamental question whether a protein suspected to control cell growth, can actually accomplish this role within a mammal. Among those proteins whose expression was targeted to mammary epithelium are growth factors, their receptors, cell cycle proteins and cellular and viral oncogenes[4] (for a summary of models see *Oncogene*, vol. 19, no. 8, February 2000). The lesson learned from these experiments is that many of these proteins can induce mammary tumors in transgenic mice. This points to a general susceptibility of mammary tissue to transformation and may be explained by the plasticity and cyclic development of this organ. With each pregnancy a functional organ originates from a population of stem cells, only waiting to be fully dismantled after weaning. Both processes require a coordinated activity of genetic pathways controlling cell proliferation, differentiation and death, and mechanisms of protease-mediated remodeling. It is therefore no surprise that transgene-mediated disruption of any of these processes can trigger transformation of the gland. This point is reinforced by several transgenic mice that carry transgenes under control of the metallothionein (MT) gene promoter. Although the MT promoter is active in most cell types, the appearance of mammary hyperplasias and tumors is a predominant phenotype in these mice.

An important and often underestimated variable in transgenic experiments is the choice of the control region used to activate the transgene. In general, the expression of transgenes is directed by either the MMTV-LTR, one of the milk protein gene promoters (WAP, β-lactoglobulin, β-casein) or the C3(1) promoter, all of which target the mammary epithelium. Although these transcriptional control elements are induced by lactogenic hormones and their expression is highest during pregnancy and lactation, their temporal activation during mammary development varies greatly. For example, while the MMTV-LTR is already transcriptionally active in ductal cells of the virgin gland, the WAP gene promoter is preferentially activated late in pregnancy in the secretory epithelium. The biological consequences are exemplified in mice that carry an *int3/notch4* transgene either under control of the MMTV-LTR or the WAP gene promoter. While MMTV-int3 mice have an early onset of tumorigenesis and do not form alveolar structures, WAP-int3 mice develop tumors later and exhibit a lobulo-alveolar compartment.[5]

Although such 'first line' transgenic experiments have been valuable and demonstrated biological capabilities of specific proteins, there are distinct disadvantages to such an approach. In addition to the differences in temporal activation of transgenes, the dependence of transgenic promoters on lactogenic hormones is a major obstacle. In order to mimic the situation of pregnancy-independent tumorigenesis it would be desirable to have a promoter at hand that is highly active in mammary epithelium in the absence of lactogenic hormones. Through efforts by the NCI CGAP and Riken, cDNA libraries from normal and neoplastic mammary tissue have been generated and sequenced in depth. This wealth of information of expressed genes should provide the basis for the identification of suitable transcriptional control elements.

Conditional transgenesis

While conventional transgenesis allows studies on tumor initiation and progression, a necessary and overdue focus needs to be on investigations of how to dismantle solid tumors in *in vivo* settings. It will be necessary to focus on biochemical and genetic events that lead to the establishment of solid tumors and are eventually independent of the oncogenic stimulus. Such studies require that the activity of an oncogenic stimulus can be controlled both in space and time. In 1992 Gossen and Bujard developed in tissue culture cells a gene switch that can be controlled by tetracycline,[6] and 2 years later Furth and her colleagues established this switch in transgenic mice.[7] Both the conventional 'tet' system and its reverse version have now been widely used to investigate the events that lead to irreversible tumor progression. In experiments that target the expression of SV-40 T-antigen to the salivary gland epithelium Ewald, Furth and their colleagues could demonstrate that T-antigen induced hyperplasias were reversible if the oncogenic stimulus was removed at 4 months of age, but that tumors were non-reversible if the oncogenic stimulus persisted for 7 months.[8] In a second model, DePinho and his colleagues could demonstrate the importance of continued RAS activation in tumor maintenance.[9] Experiments in which the MMTV-LTR

was used to control the tet system in mammary tissue were only partially successful. While expression was mosaic in mammary tissue, it was homogeneous in other secretory cells and in hematopoietic cells,[10] and these activator mice were used to study malignancies in the salivary gland[8] and B-cells.[11] Recently, Utomo and his colleagues have established transgenic mice in which the tetracycline switch is active in mammary epithelium,[12] which should help to facilitate the controlled manipulation of genes in these cells.

Synergism in bi-transgenic mice

A number of growth factors and cell survival proteins are not oncogenic by themselves, but are able to synergize with additional oncogenic stimuli. More recent studies have shed light on the interaction of oncoproteins with each other, with growth modulators and with cell survival/death signals. Specifically the synergistic role of oncogenic signals with growth regulators, such as TGFα, and cell survival signals, such as members of the bcl-2 family, has been investigated. For example, the cell survival molecule bcl-2 does not induce hyperplasias and neoplasias when expressed as transgene in mammary epithelium by itself.[13] However, its presence reduces apoptosis in mice that express the myc oncogene[13] and the SV-40 T-antigen in mammary epithelium and it blocks the cell cycle, which in itself results in an accelerated tumorigenesis.[14] Furthermore the antiapoptotic action of the bcl-2 transgene persists throughout tumorigenesis.[14] Similarly, the absence of Bax by itself does not result in profound changes of mammary development and pathology. In addition, neither the absence of one or both bax alleles by itself accelerated tumorigenesis in transgenic mice expressing T-antigen in mammary tissue.[14] However, the additional introduction of a bcl-2 transgene resulted in an earlier appearance of tumors,[14] suggesting that a gain of bcl-2 can synergize with a loss of bax function. In another transgenic model for mammary cancer in which the SV40 T-antigen is expressed under the C3(1) promoter, the role of bax appears to be slightly different. In this model, loss of one bax allele resulted in a higher incidence of tumors at 19–21 weeks of age despite no change in tumor incidence before or after this timepoint.[15] This again emphasizes that temporal and/or spatial activation of a transgene and possibly strain difference can modulate the physiological effects of genes.

Mammary pathology of genetically engineered mice

Although many mouse models have been championed as recaptulating key features of human breast cancer, few have been validated and compared directly with human tissue. The National Institutes of Health decided that the accurate pathologic analysis of mammary lesions in genetically engineered mice is critical to progress in understanding these models, and convened a workshop in Annapolis in 1999. A panel of nine surgical, veterinary and experimental pathologists convened in Annapolis and was asked to compare the pathology and classification of mouse and human mammary cancers. The panel evaluated tissues from 39 mouse models and the consensus report and recommendations have been published[4] (`http://mammary.nih.gov/Annapolis-guidelines`). One of the major observations at the workshop was that the medical and veterinary pathologists do not necessarily have a common vocabulary to describe or name specific lesions. The language gap is even worse between the pathologist and the mammary and molecular biologist.

In comparing the biology from human breast tumors to mammary tumors in genetically engineered mice, the Annapolis pathologists identified similarities and differences. Among the similarities are: (1) molecular lesions causing breast cancer in humans can also cause cancer in transgenic mice; (2) the lesions in both species display similar morphological patterns; (3) multi-hit kinetics of cancer development; (4) mammary cancers in both species are metastatic; (5) mammary cancer is frequently hormone independent. Among the differences are: (1) some molecular lesions causing mammary cancer in mice have not been found in human mammary cancer; (2) the morphology of most mouse tumors does not resemble the common human cancers; (3) some transgenes in mouse appear to be associated with a single hit kinetics; (4) most mouse tumors metastasize to the lung, while most human tumors metastasize to the lymph nodes; (5) half of the human cancers are hormone independent, but most mouse tumors are.

The meeting organizers felt that the scientific community would benefit from the Annapolis slide set. However, the slide collection could not be shared with every laboratory doing mammary tumor research. Further, publication of a limited set of images in the standard journal format would

not provide the scientific community with these wonderful resources. As an alternative, funding was obtained from the Cancer Genome Anatomy Project (CGAP), NCI and NIDDK to develop the enclosed CD-ROM. The CD-ROM includes examples of normal growth and development, non-neoplastic lesions, and breast cancer. Instructions, with examples on techniques such as whole-mount preparation, immunohistochemistry, *in situ* hybridization, and common histological stains are provided. Images are annotated and mouse models have an accompanying citation. Tables are provided for orientation and organization. The CD-ROM includes zoom capabilities, a search engine, and a help mode. Above all, this CD-ROM is an experiment to the future of publishing. It is a bold attempt to provide the readership with modern multimedia advantages. The images are all based on fullscale 1996 × 1640 pixel images at 300 pixels/inch. The resolution exceeds that of any current electronic journal page and provides various 'zoom' options for high-resolution viewing. Some images and text are 'outlinked' for those who wish to use the Internet to 'drill down' for greater detail. In addition to the CD-ROM, an interactive web-based histology atlas was established that contains the Annapolis images (`http://HistoBank.nih.gov`).

Moving forward

The tidal wave of transgenic studies has provided a wealth of information on molecular pathways and cancer physiology. However, these studies have also revealed problems inherent to transgenic mice and technical challenges have to be met. The challenges come in different categories, which include inherent biological differences between mouse strains, the diverse expression patterns of transgenes, the development of new technologies to control multiple genes simultaneously and the identification of promoter systems that can be activated preferentially in mammary epithelium and stroma independent of lactogenic hormones.

Strain differences

There is no doubt that the nature of the mouse strain can greatly influence development and physiology of the mammary gland, and the latency and even the type of the tumor caused by a transgenic oncoprotein. This was not an apparent problem in the early days of transgenesis (mice were generated in only a few inbred backgrounds and in C57BL/6 × SJL hybrids) when investigators studied mice carrying individual transgenes. However, more recently investigators have studied mice, which carry several transgenes and gene deletion mutants. This resulted in the introduction of the 129 strain background. Hormonal signaling pathways in the mammary gland seem to be particularly sensitive to strain-specific differences. For example, mice that carry one mutant and one wild type allele of the prolactin receptor cannot lactate in a C57BL/6 background, but develop a functional mammary gland in a 129 × C57BL/6 mixed background.[16,17] There is concerted effort by investigators and centers, such as the Jackson Laboratory, to backcross transgenic and gene knock-out strains into the 129 and C57BL/6 backgrounds, accelerated through the use of speed congenics. The discovery of distinct strain differences also provides an opportunity to identify modifier genes in a defined setting that is not possible in humans. The power of such systems has been demonstrated with the Min/APC locus.

Cell-specific, hormone independent promoters

At this point the choice of promoters to target transgenes to the mammary gland is restricted to those that can be activated in the epithelium by lactogenic hormones. To address questions, such as the role of the stroma during development and tumorigenesis and whether pregnancy provides limited protection from breast cancer, it is necessary to discover and develop transgenic control elements that can be activated in different tissue compartments (epithelium versus stroma) and independent from hormonal stimuli. Again, through NCI CGAP efforts and the availability of extended Riken libraries it should be possible to identify Ests that are preferentially found in the stromal compartment of the mammary gland, and thus isolate the respective DNA control elements.

Conditional gene targeting

The most important application, perhaps, of gene targeting in cancer research is the possibility to reliably explore the interaction of oncogenic stimuli with endogenous signaling pathways—many of them of hormonal nature. Traditional gene knock-out experiments based on ES gene targeting have been extremely useful in identifying gene function in mammary gland development and oncogene-mediated

tumorigenesis within the mouse. For example, the contribution of p53 to tumor progression has been studied in p53-null mice carrying different oncogenes. However, there are limitations to this technology. In many cases the physiological consequence of the gene deletion makes it impossible to study mammary tissue. In certain cases, the deletion of a gene results in fetal lethality (e.g. Rb1, Brca1, bcl-x) or infertility (Stat5ab, estrogen and prolactin receptors). Further, global gene deletion does not easily permit investigations of cell autonomy and distinguish local alterations from systemic effects. Lastly, only early consequences resulting from the loss of a protein can be observed. Several remedies are already being offered. Among them is the conditional deletion of genes in defined cell types. For example, global deletion of the Brca1 gene results in embryonic lethality, but the mammary cell specific inactivation using Cre-loxP based recombination permitted studies on the role of Brca1 in tumorigenesis.[3] However, the problems encountered with the currently available promoters, as discussed earlier, will remain the same or will even be exacerbated. Although the MMTV-LTR targets transgene expression preferentially to mammary epithelium as shown in conventional transgenic experiments, low level expression can be seen in many epithelial cell types and the hematopoietic compartment. Since even a transient expression of Cre results in a permanent deletion of a gene flanked by loxP sites, physiological consequences will be seen in a variety of cell types.[18,19] The integration of the tetracycline switch with the Cre-loxP recombination system will further permit the temporal and the cell-specific deletion of genes.[12] Utomo and colleagues generated transgenic mice that carry the reverse tetracycline time switch under control of the WAP gene promoter, which should enable them to keep a transgene silent until the mouse is exposed to tetracycline or a derivative thereof.

If the deletion of the gene under investigation results in infertility or lethality after embryonic day 13.5 it is possible to nevertheless study mammary epithelial cells without using Cre-loxP based cell-specific recombination approaches. It is possible to isolate mammary epithelium from the mammary anlage or any stage thereafter and rescue it upon transplantation into the stroma of wild type mice.[20] Such experiments, for example, will permit the analysis of pRb-deficient mammary epithelium. In addition, tissue recombination experiments permit studies on the role of genes in distinct tissue compartments, such as the epithelium and stroma.[21]

Obstacles are also being faced in studies that address the role of some hormones signaling pathways in mammary cancer. It is clear that hormones, such as prolactin, growth hormone and epidermal growth factor signal through Stat5 and possibly modulate tumor progression.[22] However, conventional transgenic promoters used to target genes preferentially or exclusively to the mammary gland probably cannot be used in experiments addressing the role of these hormones in tumor progression. Mice in which the Stat5 or prolactin pathway has been disrupted contain some mammary epithelium, which, however, is undifferentiated. Therefore, the available transgenic control elements will not efficiently activate oncogenes in this background. Again, there is a need for promoters that are active preferentially in mammary epithelium but do not depend on the presence of lactogenic hormones.

Great advances have been made to decipher genetic pathways controlling normal and neoplastic development of the mammary gland. The wisdom gained and the biological challenges ahead should inspire investigators and funding agencies alike to further invest in the tools of mouse genetics and biochemistry. We are on the verge of understanding the ground rules underlying mammary gland development, a starting point to develop better mouse models that reflect human breast cancer.

Acknowledgements

I thank my laboratory for keeping up the intellectual and technical challenges, Bob Cardiff for his continued encouragement to develop better teaching tools and Priscilla Furth for providing the focus.

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